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14. ABSTRACT: The objective of this study was to discover how we can enhance the immunity to tumor Antigens (TA) , in the cancer vaccines. Preventive immunity to cancer in high risk patients or in patients with primary disease is important. The importance rests with a better quality of life and decrease medical costs. To reach the immunopreventive level a vaccine should activate strongly immunity to TA. The strong activation consists in expansion and differentiation of T cells specific for TA. The more effectors expanded by vaccine, the more memory cells. The more differentiated the T cells, the more functional. Our hypothesis is that optimization of the contacts: TA -T cell receptor (TCR) contacts will activate more T cells and it will induce their differentiation. We focused on the side chains of the amino acids in the tumor A. We avoided the changes in the peptide core to minimize cross-reactivity. We discovered that: (1) if side chains are introduced in the amino acid glycine, then the immunogenicity of the TA increase following a bell-shaped plot. (2) if side chains which form the points of contact(link) TA-T cell receptor are replaced with others which induce electrostatic repulsion, then activation of cells is attenuated. The attenuation minimized death of "memory –like "cells. (3) Increase in distance between TA and TCR with one CH2 group also increase survival of cells activated by TA. (3). Changes in the length and orientation of the N-terminus of the TA also result in increased activation of T cells. The N-terminal variants are effective at low concentrations, but induce death by over-activation. Additional studies were performed towards future goals. The findings of this study were recognized for joint technology elopment and commercialization by MDACC and the Henry Jackson Foundation.

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Key words: CTL, tumor antigen, hydrophobic appendage, T cell receptor, tumor immunity, humans, breast cancer, ovarian cancer.
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Introduction

Human $CD8^+$ cells expressing higher-than-average numbers of TCR (TCR^{hi}) reportedly have high functional avidity for their tumor targets (1-3), as demonstrated by their ability to lyse targets pulsed with smaller amounts of exogenous Ag (10^{-7}–10^{-8} M) than do CTLs expressing fewer TCR.

This is why they are called high-avidity *(hi-av)*. T cells. B cells differentiate at re-stimulation by changing the use of the heavy chain gene (from m to g) and aquiring mutations in the junctional regions. T cell receptors do not mutate at restimulation but two things happen. One is that T cells bearing some receptors expand , the other is that T cells bearing other receptors die. Because the hi-av cells are also high-TCR density cells, they are more sensitive to death than low-avidity(low-density cells). These observations suggest that high TCR density compensates for the low affinity of individual TCRs for self-Ag. TCR^{hi} CTLs, the most potent cytolytic effectors identified so far, are scarce in patients with cancer (1-3). In one study, some TCR^{hi} cells were shown to be insensitive to Ag or died at Ag concentrations of 10^{-6} M (3); in another study, TCR^{hi} cells expanded in response to Ag but required IL-7 and IL-10 for survival (2). These findings suggest that tumor Ag transmitted a negative signal to block the differentiation of those CTLs and that the cytokines used for CTL expansion apparently amplified the negative signal. In ligand: receptor interactions, when the concentration of the ligand is constant, the effects of the ligand on the cell depend on the density of its receptor. At constant ligand concentration and constant receptor density, the functional effects of the ligand change with its affinity for the receptor or the duration of the receptor engagement.

To elucidate the significance of TCR density in the differentiation of TCR^{hi} cells to cytolytic effectors we modified the affinity of the ligand (Ag) for the receptor (TCR) and simultaneously analyzed the responses of two polyclonal $CD8^+$ populations from the same individual that differed in the levels of TCR by one order of magnitude. Because the transition between mitosis and apoptosis in TCR^{hi} cells responding to tumor Ag takes place within a narrow range of Ag concentrations (5×10^{-6} M -10^{-7} M) (3), we modified the affinity of the Ag for TCR in the smallest possible changes (increments/decrements), using only atomic van der Waals forces from methylene (CH_2) groups appended to Gly. At only 2 kJ (0.5 kcal) per mole, van der Waals forces are the weakest forces between atoms; by comparison, the force involved in the formation of one hydrogen bond after the introduction of one hydroxyl group is 20 kJ per mole (4). Since longer and branched side-chains produce steric hindrance, and steric hindrance may offset gains from increments in van der Waals forces, we used only short linear CH_2 extensions.

We used CTLs isolated from tumor-associated lymphocytes (TALs) that recognize an epitope from the HER-2/*neu* protooncogene, which is present on normal epithelial cells but is also overexpressed in many epithelial cancers of the breast, lung, prostate, and ovary (5). Because HER-2 is a self-Ag, most T cells of high affinity for HER-2 epitopes are deleted (6), and the remainder recognize HER-2 peptides with low avidity (at about 10^{-6} M). Nevertheless, because the same HER-2 CTL epitopes are presented by substantial proportions of tumors, the CTL epitopes from HER-2 become significant for cancer therapy (7).

How undifferentiated TCR^{hi} cells respond to human tumor Ag and what is needed to induce their proliferation and differentiation to functional effector cells remain unknown. To identify the optimal agonist for inducing differentiation of TCR^{hi} cells, we constructed four variants of E75, the HER-2 (369-377) epitope for CTLs, by appending one, two, three, or four methylene groups to the glycine molecule at position 4 (Gly^4) to form a linear C-side chain. We then selected cells expressing high concentrations of TCRs for E75 (E75-TCR^{hi}) to evaluate the role of TCR density in CTL differentiation upon stimulation with the same ligands. The TCR^{hi} population which usually includes cells staining with Ag-tetramers/dimers with a mean fluorescence intensity (MFI) higher than 10^2 was divided in two populations, one staining with Ag-pulsed HLA-A2: IgG dimers (dimers) with a MFI (TCR) between 10^2-

10^3, and another which stained with Ag-pulsed dimers with a MFI (TCR) between 10^3-10^4. These populations were designated as TCR^{med} and TCR^{hi} respectively.

The ability of CTLs to synthesize perforin is critical to their ability to lyse target cells (8,9). Presumably cells with high-density TCR would need correspondingly high amounts of effector molecules such as perforin for maximum functionality. Perforin is undetectable in naïve CTLs but is up-regulated in response to signals from TCRs (8-10). Perforin also controls $CD8^+$ homeostasis independently of its role as an effector molecule (11-13). Large expansion of Ag-specific $CD8^+$ cells that produce IFN-γ in perforin-deficient mice resulted in lethal disease during viral infections (11,14). Most T cells with high affinity for self-Ag are deleted upon encountering that self-Ag; that deletion constitutes a mechanism for protection against autoimmunity (6,15). We sought to determine how to induce differentiation of such cells and to identify the factors controlling their differentiation and survival. The difficulty in addressing these questions is compounded in polyclonal human systems, not only because surviving TALs recognize Ag with low functional avidity because their perforin expression is impaired (16,17), but also a large number of TCRs with distinct affinities for Ag are present.

BODY

Our proposed objectives (Aims) were as follow:

Aim 1. *To optimize induction of hi-av effector CTL by modifying the van der Waals capability of E75 (A7.0) and of its analog A7.3.*

Aim 2*. To optimize induction of memory tumor reactive hi-av CTL using attenuated HAB Ag.*

Aim 3*. To characterize the molecular basis of HAB Ag induced survival of memory CTL.*

The DOD review pannel noted that the proposed tasks in the Aim 3, were premature and over ambitious. The review panel recommended the applicant to focuss on the Aims 1 and 2. Since funds were available from other sources, we extended the study below). Furthermore since new administrative procedures required that the grant funds be used for payment of salaries for certain positions additional work was performed on topics which constitute future developmet of this project. These topics are: expression of the tumor Ag in small RNA vectors and identification of self-immuno-stimulatory multimolecular complexes. (ISMMC).

Materials and Methods.

Cells, Antibodies, and Cytokines. The ovarian TAL line TAL-1 was generated from heparinized ovarian ascites, collected under institutionally approved protocols. Lymphocytes from TAL-1 were cultured in t he presence of low concentrations of IL-2 (150-300 IU/ml) for 7 –14 days. Stimulation of these TAL-1 cells with 200–1,000 nM of any Gly^4 variant resulted in their secreting IFN-γ at levels increasing in tandem with the length of the CH_2 chain, indicating that the E75-TCR^+ cells in the TAL-1 population were functional and not tolerized. (not shown)mAb used for detecting surface Ag and cytokines used for culturing T cells from TAL-1 are described elsewhere (18). APC-conjugated antibody to IFN-γ (IgG2a), PE-conjugated mouse (IgG2b) antibody to perforin (δG9), empty recombinant soluble dimeric human HLA-A2:IgG1 designated as “dimers”, and all specific isotype Ig controls were obtained from BD Pharmingen (San Diego, CA). Proteasome and caspase inhibitors were obtained from R&D Systems (*Minneapolis, MN).*

Synthetic Peptides. The peptides used were E75 [HER-2 (369-377); KIFGSLAFL] (19) and its methylene-appended C-side-chain variants. Four CH_2 variants were generated by substituting the glycine at position 4 (Gly^4) with alanine and one of following synthetic amino acids: γ-aminobutyric acid, norvaline, or norleucine. **(** Advanced Chemtech, Louisville,KY) The abbreviations for the CH_2-extended E75 variants (G4.1, G4.2, G4.3, and G4.4) reflect the position (Gly^4) and number of the CH_2 group extensions (19). Another variant in which the alanine at position 7 (Ala^7) was replaced with norleucine was designated A7.3. All peptides were prepared by Dr. Martin Campbell (Peptide Synthesis Core Facility of The University of Texas M. D. Anderson Cancer Center). Amino acids were coupled in sequential format from the C-terminus using standard FMOC peptide chemistry on a Rainin Symphony Automated Peptide Synthesizer and purified by HPLC. The purity of the peptides ranged from 95% to 97%. Peptides were dissolved in PBS and stored at –20°C as aliquots of 2 mg/ml until use. Molecular modeling of peptide: HLA-A2 complexes was performed by Dr. Darrick Carter using as model the crystal structure of Tax peptide bound to HLA-A2 .

T Cell : Peptide-HLA-A2 Dimer Association and Dissociation Assays. Expression of TCRs specific for HLA-A2 bound to the E75 peptide (E75-TCR^+ cells) was determined by using E75 dimers (dE75). dE75 were prepared as previously described(18) . Staining of cells was performed as described previously (19-21). Specific geometric (y^2) MFI (TCR) for each peptide was calculated by substracting the MFI (TCR) of cells stained with "empty dimers" (dNP) from the MFI (TCR) of cells stained with dE75, dG4.1, dG4.2, dG4.3 and dG4.4. To identify the changes in the affinity of variants for TCR, the y^2 (MFI) of TAL-1 cells stained with each peptide dimer, was determined immediately after staining (t_0) or two hours later (20-23**).** Empty dimers were prepared in the same conditions as dE75 dimers excepting that no peptide was added to the HLA-A2-IgG dimers. The increase or decrease in the MFI (TCR) induced by each CH_2 group extending the CH_2 chain relative to E75 was calculated by subtracting the MFI (E75-TCR) from MFI (TCR) of cells stained with each variant peptide (G4.1, G4.2, G4.3 and G4.4) and dividing the result by the number of CH_2 groups appended to each variant (1,2,3,4). These values were designated as MFI (TCR) per appended CH_2. Among cells staining positive with peptide bound to HLA-A2: IgG dimers, populations were considered to express TCR at low density (TCR^{lo}) if the geometric (y^2) MFI for cells staining with that dimer was between 10^1 and 10^2, at medium density (TCR^{med}) if the MFI was between 10^2 and 10^3, and at high density (TCR^{hi}) if the MFI was between 10^3 and 10^4.

T-Cell Stimulation by the CH_2 Variants. Apoptosis was induced in TAL-1 by two successive stimulations with 10,000nM of each peptide pulsed on T2 cells. Surviving TAL-1 were then primed with 5,000nM of each peptide pulsed on irradiated T2 cells as described elsewhere (24, 25). Control cultures were stimulated with T2 cells that had been pulsed with peptide A7.3 . The death-resistant cells were then re-stimulated *in vitro* with irradiated T2 cells pulsed with each peptide at a final concentration of 5 μM and expanded in IL-2. Expression of IFN-γ and perforin (Perf), markers of differentiation, was determined by using IFN-γ-APC–conjugated or perforin-PE–conjugated antibodies and matched PE/FITC/APC-conjugated isotype controls on dE75-stained and permeabilized cells (18). The fold expansion by each variant was calculated by dividing the number of E75-TCR^{hi}, TCR^{med} cells detected in each sample after stimulation by the number of E75-TCR^{hi}, TCR^{med} cells present before stimulation.

CTL Assays. E75-, A7.3-, and G4.1- to G4.4-stimulated TAL-1 were used as effectors in CTL assays. Ag recognition by the E75-variant-induced CTLs was determined as described elsewhere (26, 27). Recognition of E75 was considered specific when the mean specific lysis of T2 cells pulsed with E75 minus the standard deviation (SD) was at least 10% and was at least twice as high as the percentage of specific lysis of T2 cells that had not been pulsed with peptide, plus the SD (27) E75-specific tumor lysis was determined by subtracting the levels of SKOV3.A2 tumor lysis observed in the presence of T2-E75 cells from the levels of SKOV3.A2 tumor lysis observed in the presence of T2-NP cells (27). The tumor cells were then incubated with 10 μM MG132 (28, 29) for 30minutes, before and during labeling, then used as targets in CTL assays. High- and medium-avidity effector CTLs were

distinguished in two ways-first by their ability to recognize E75 at concentrations at least two times lower than the E75-primed CTL (e.g., at 500 nM instead of at 1000 nM) and second by the ability of high-avidity CTLs to mediate an effector response (e.g., % specific lysis) that was (a) at least twice as high as the effector response at the same or lower Ag concentration and/or (b) at half the effector -to-target ratio of the medium-avidity CTLs.(30)

Caspase Inhibitors. The caspase inhibitors Z-IETD-fluoromethyl ketone-(fmk) (specific for caspase-8), Z-LEHD-fmk (specific for caspase-9), and EDVE-fmk (specific for caspase-3), have been reported to participate in perforin-mediated apoptosis (31). For these experiments, 2 x 10^6 G4.2-induced CTL were incubated with each caspase inhibitor at 37°C for 90 minutes, washed twice with PBS, and then stimulated with T2 cells pulsed with 5 μM of G4.2.

Statistical Analysis. Differences in the levels of IFN-γ, perforin, and cytolysis were compared by using unpaired Student's *t*-tests from triplicate determinations. Differences were considered significant at $P < 0.05$.
Standard methods such as Western Blotting are described in the attached publications (references 31,32)

RESULTS
A large amount of results were already published. Most of the related publications are attached. In the final report we we summarize our published findings. We also describe in more detail the methods and results which were not published yet.

Aim/Task 1. *To optimize induction of hi-av effector CTL by modifying the van der Waals capability of E75 (A7.0) and of its analog A7.3.*

Extending Peptide Side Chains with CH_2 Modifies the Affinity of E75 for TCR. Molecular modeling of the E75-HLA-A2 complex indicated that CH_2 extension in Gly^4 resulted in zigzag orientation of the CH_2 chain towards the solvent Peptide: HLA-A2 association and dissociation assays indicated that appending CH_2 groups did not increase the affinity of the variants for HLA-A2 over that for E75 and the stability of peptide: HLA-A2 complexes. **(**previous report**)** Peptide-HLA-A2 IgG dimer: TAL-1 association and dissociation assays, done to determine how the CH_2 appendages affected the affinity of the peptide-HLA-A2 complex for TCR, showed that at t_0, TAL-1 stained more strongly with dG4.2, dG4.3, or dG4.4 than with dE75 or dG4.1, meaning the ligands G4.2, G4.3 and G4.4 had higher affinity for TCR . dG4.1 staining was weaker than dE75 staining. G4.1 dissociated faster than E75, G4.2 dissociated slightly slower than E75, while G4.3 and G4.4 dissociated slower than G4.2. The MFI (TCR) per CH_2 group appended followed a bell-shaped plot with a peak at G4.3. The overall specific affinity for TCR of each variant increased with addition of CH_2 groups but showed saturation at G4.4 . Changes in the MFI confirmed that each CH_2 group in the G4.1–G4.4 variants interacted with the TCR; moreover, each CH_2 group added to the chain affected the interaction of the existing CH_2 groups with the TCR and changed the affinity of the other groups for TCR and the stability of the TCR: peptide HLA-A2 complexes**.** Results show the average change in MFI (TCR^{hi}) and MFI (TCR^{med}) per added CH_2 group at t_0 (0h) and 2h later (2h). Both MFI (TCR^{hi}) and MFI (TCR^{med}) formed bell-shaped plots which peaked with G4.3 and G4.2 respectively. These results indicate that affinity of variants for TCR increased only within a range. In summary, except for the single-methylene-group variant G4.1, CH_2 extension increased the binding affinity of the variant for TCR^{hi} without increasing the binding affinity for HLA-A2.(see reference 32), also attached publication.

Priming Apoptosis-Resistant TCR^{hi} IFN-γ^- $Perf^-$ TAL-1 with E75 or its Gly^4 Variants Induced Their Differentiation to IFN-γ^+ $Perf^-$ Cells. TAL- 1 contained significant proportions of *ex vivo* activated and differentiated T cells. TCR^{hi} and TCR^{med} cells were of similar size (mean forward scatter, 630), indicating that they were activated, not resting, cells and that the higher TCR density was not related to cell size. Representative results for $TCR^{hi \text{ and } med}$ expression, cell size, and staining for perforin-

positive (Perf $^{+}$) and IFN-γ^{+}-positive cells before apoptosis-induction, after the first stimulation and after the second stimulation are shown in reference 32. To recapitulate the process of differentiation, we first deleted differentiated cells, i.e., TCR^{hi} and TCR^{med} Perf^{+} IFN-γ^{+} cells by stimulating TAL-1 twice with 10,000 nM E75, G4.1, G4.2, G4.3, or G4.4 pulsed on T2 cells to ensure that most (if not all) TCR^{hi} Perf^{+} IFN-γ^{+} cells were eliminated. E75 and all of the Gly^{4} variants with higher affinity for TCR than E75 deleted most of the TCR$^{hi\ and\ med}$ Perf^{+} cells and most of the IFN-γ^{+} cells (also appendices). A7.3, the CH_2 position control peptide for G4.4, also induced deletion of TCR^{hi} Perf^{+} cells. Differences between the small numbers of surviving cells were not significant. The numbers of TCR^{hi} Perf^{+} cells decreased insignificantly after stimulation with control T2 cells, which present only few endogenous self-peptides compared with unstimulated IL-2 cultured TAL-1.

Surviving cells were then primed with 5,000 nM Ag (i.e., half the concentration that induced apoptosis, which led to deletion of TCR^{hi} Perf^{+} cells) , leaving only TCR^{hi} Perf^{-} cells. To facilitate comparisons, the results are shown reported to 10^{6} E75-TCR^{+} cells (E75-TCR^{hi} and E75-TCR^{med} cells. Only IFN-γ^{+}, Perf^{-} TCR^{hi} cells were present. Although the majority of TCR^{hi} and TCR^{med}, Perf^{+} died, some of the cells continued to express IFN-γ.

Priming with variants did not significantly increase the numbers of E75-TCR^{hi} cells. The ratio of E75-TCR^{med} to E75-TCR^{hi} which was 6:1 in control T2-NP cultures, remained the same (E75), or increased in the cultures stimulated with A7.3 and G4 variants. E75-TCR^{hi} cells stimulated with E75, A7.3, and G4.1 expressed perforin, while cells primed with G4.2, G4.3 and G4.4 did not. In contrast, the majority of E75-TCR^{med} stimulated with G4.2, G4.3, and G4.4 expressed perforin, while few E75-TCR^{med} cells stimulated with E75, A7.3, and G4.1 expressed perforin. It is unclear whether the E75-TCR^{hi} Perf^{+} cells derived from the few Perf^{+} cells which survived apoptosis or from the Perf^{-} cells which differentiated in response to Ag-stimulation. It is evident that stimulation with variants had different effects than stimulation with wild-type E75, in perforin and IFN-γ induction, in E75-TCR^{hi} cells and E75-TCR^{med} cells. Results indicated that CH_2 appendage was effective in inducing differentiation of both TCR^{hi} and TCR^{med} cells, but the effects differed, depending on the position of the appendage (G4 or A7) and the density of the TCR. We quantified the effects of stimulation with variants of polyclonal populations only on the cells expressing E75-TCR. The effects of the variants on cells expressing specific TCR for the variants, or reacting with the variants with higher affinity than with E75 have not been determined. Therefore, it may not be excluded that e.g. E75-TCR^{med} cells are also G4.2-TCR^{hi} cells. The patterns of specific MFI (TCR) for variants-dimer complexes show parallel or inverse relationships with the resulting E75-TCR^{med} Perf^{+} cells.

To identify the effects of restimulation in E75-TCR^{+} cells differentiation, all cultures were restimulated with T2 cells pulsed with the same amounts of priming peptide, as in vaccination studies. Results show a significant increase in the numbers of TCR^{hi} cells, which was paralleled by a significant decrease in the numbers of E75-TCR^{med} cells. The decrease in E75-TCR^{med} cells ranged between 75-96% for cells stimulated by E75, G4.2, G4.3, and G4.4 respectively. E75-TCR^{med} cells increased by 45% in cultures restimulated with G4.1. In contrast, E75-TCR^{hi} cells increased almost eight-fold in cultures restimulated by E75 and G4.3, and by 15-20 fold in cultures restimulated with G4.1, G4.2 and G4.4. Because these populations are polyclonal, we cannot distinguish whether expansion of TCR^{hi} cells was due to a higher rate of division of only TCR^{hi} cells or to a 3-4 fold increase in the numbers of E75-TCR molecules per cell in populations of E75-TCR^{med} cells, or both. The increase in E75-TCR^{hi} cells in cells stimulated with G4.1, of lower affinity for TCR than E75, is surprising and suggests a process similar with homeostatic proliferation and differentiation induced by low affinity ligands.

G4.2 is the Most Effective in Inducing Differentiation TCR^{hi} Cells to Perf^{+} Cells. Perforin expression has been associated with terminal differentiation of CTL . If TCR^{hi} cells are partially differentiated, they would express perforin at restimulation; on the other hand, if they cannot differentiate, they would die at restimulation. To address this question, we examined perforin expression in primed and restimulated E75-TCR$^{hi\ and\ med}$ cells. The levels of perforin increased only slightly at restimulation. In

contrast, E75-TCR^{hi} cells expressed very low levels of perforin at priming which increased at restimulation. The levels of perforin increased by 5-fold in cultures restimulated with E75 and G4.3 and by 7-fold in cultures stimulated with G4.1. G4.2 and G4.4 induced the highest levels of perforin in cells which lacked perforin after priming. We cannot exclude the possibility that some of the E75-TCR^{med} cells increased both the number of TCR molecules/ per cell and the amount of perforin per cell and become E75-TCR^{hi} cells. Of interest, many E75-TCR^{hi} cells, restimulated by E75, G4.1 and G4.3 became apoptotic; the effects were stronger for G4.3 followed by E75 and G4.1 (data not shown).

CTLs Induced with G4.2 Lyse Tumor Cells. To address whether variant activated CTL had higher functional avidity for E75 than E75-activated CTL, we assessed the recognition of E75 by variant-induced CTLs. In the first experiment effectors were variant-primed cells, and only the TCR^{med} populations expressed perforin. Only G4.4-CTLs significantly recognized E75 (>10% lysis) at concentrations of 100 and 500 nM while lysis by G4.2 CTL was below the 10% cut-off level to be considered significant. The levels of perforin IFN-γ (G4.2: MFI (IFN-γ) = 25.9, G4.4 MFI (IFN-γ) = 18.23), E75-TCR (G4.2: MFI (y^2)= 212, G4.4 (MFI (y^2)= 158 were similar in G4.2 and G4.4 primed cells. Thus the results suggest a better "fit" between E75 and CTL primed by G4.4 resulting in higher functional avidity. E75-CTL did not recognize E75 at this concentration, but rather required 2,500 nM of the peptide for lysis to be detected (not shown).

Therefore G4.2-expanded TCR^{hi} Perf^{+} cells of higher functional avidity for E75 than G4.4 and G4.1. The increase in functional avidity of the G4.2-CTL also paralleled the two-fold increase in perforin levels in cells expressing one log higher TCR levels.Death of G4.1 stimulated cells eliminated CTL which recognized E75 with higher affinity in this experiment. Specific lysis by G4.1-stimulated CTL for 500nM E75 decreased to 5% in a second experiment (not shown).

These results indicate that re-stimulation with G4.2 expanded better CTL of higher functional avidity for E75. These CTL were also more stable and survived longer than CTL stimulated with E75, G4.1, and G4.3.

To determine whether the higher functional avidity for E75 reflected a high functional avidity for tumor, we assessed E75-specific tumor lysis in E75-blocking experiments. In these experiments, SKOV3.A2 cells were treated or not treated with IFN-γ to activate Ag presentation. To verify that E75 was being processed endogenously, we treated targets with the proteasome inhibitor MG132 before adding the effectors. The IFN-γ-treated SKOV3.A2 cells were more sensitive to G4.2-CTL in the 4-hour CTL assay than were the untreated SKOV3.A2 cells. Lysis of IFN-γ-treated tumor cells by G4.2-CTL had continued to increase at 20 hours, demonstrating that G4.2-CTL had high and stable functional avidity for E75. MG132 inhibited SKOV3.A2 lysis by 60% in the 4-hour CTL assay, indicating that most of the E75 was being processed by proteasomes.

Aim 2. *To optimize induction of memory tumor reactive hi-av CTL using attenuated HAB Ag*

1. Re-activation by G4.2 in the presence of Caspase-9 inhibitor increases the numbers of TCR^{hi} Perf^{hi} cells. Induction of TCR^{hi} Perf^{+} cells raised the question of whether these cells were sensitive to Ag-induced apoptosis and, if so, how to avoid that Ag-induced cell death. To identify the pathway of preferential deletion by Ag of Perf^{+} cells, expressing high levels of perforin, the G4.2 cells used in the previous experiment were "*rested"* by culturing them in the absence of IL-2. Rested cells *in vitro* for various time intervals in the absence of IL-2 or other Beta-chain cytokines represent the "memory-like cells"

T cells were treated with an inhibitor of caspase-8 or caspase-9, or remained untreated, and then stimulated with G4.2. IL-2 was added 24 hours later to avoid interference with TCR stimulation. Perforin expression was measured 48 hours later in E75-TCR^{hi} Perf^{+} cells, and as an internal control in E75-TCR^{med} Perf^{+} cells. Although separation of these cells at MFI (10^3) is arbitrary, from the levels of perforin, two populations were clearly distinguished in both TCR ^{hi} and TCR ^{med} cells: one population expressing MFI (Perf) < 100, and a second population expressing MFI (Perf) > 300.

Pretreatment with the caspase-8 inhibitor increased the number of E75-TCR^{hi} cells by only 15% and decreased their perforin level by 10% compared with cells stimulated with G4.2 in the absence of caspase-8 inhibitor. By contrast, treating the G4.2 cells with the caspase-9 inhibitor doubled the number of TCR^{hi} cells and produced a 50% increase in perforin level per cell relative to stimulation with only G4.2 .These findings indicate that G4.2 induced death in TCR^{hi} Perf ^{hi} cells by activating caspase-9. In contrast, in E75-TCR^{med} cells, the caspase-8 inhibitor was more protective than the caspase-9 inhibitor, increasing the numbers of E75-TCR^{med} cells by 65% as opposed to only 25% for the caspase-9 inhibitor relative to the cells, which were not pretreated with caspase-inhibitor However the levels of perforin, in **the** Perf ^{hi} population, increased by 21% in the caspase-9 inhibitor-treated population, suggesting that caspase-9 is activated by TCR to delete cells expressing high levels of perforin.

2. Weaker side-chain modified agonists than E75 prolong survival of E75-induced CTL.

1. We modified E75 at the central Ser5 (E75 wild-type), which points upward, by removing successively the HO (variant S5A) and the CH2-OH (variant S5G). Replacement of the OH with an aminopropyl (CH2)3-NH3 (variant S5K) maintained a similar upward orientation of the side chain. S5A and S5G were stronger stimulators while S5K was a weaker stimulator than E75 for induction of lytic function, indicating that the OH group and its extension hindered TCR activation.

S5K-CTL survived longer than did CTL induced by E75 and the variants S5A and S5G, which became apoptotic after restimulation with the inducer. S5K-CTL also recognized E75 endogenously presented by the tumor by IFN- production and specific cytolysis. S5K-CTL expanded at stimulation with E75 or with E75 plus agonistic anti-Fas mAb. Compared with S5K-CTL that had been restimulated with the inducer S5K, S5K-CTL stimulated with wild-type E75 expressed higher levels of E75-TCR + and BCL-2. Activation of human tumor-reactive CTL by weaker agonists than the nominal Ag, followed by expansion with the nominal Ag, is a novel approach to antitumor CTL development.

Apoptosis resistance in stimulated T cells at day 4 is mainly due to the intrinsic pathway. Note that these were not Ag-primed cells, but primed and restimulated.
Because resistance to Fas-induced apoptosis was suggestive of TCR-induced protection, we investigated the effects of E75 and S5K in up-regulation of Bcl-2, Bcl-xL, and Bad. Unstimulated and DC-NP-stimulated CD8+ cells from S5K-CTL were used as negative controls, while S5K-CTL stimulated with the agonists A7.3 and F8-1 were used as positive controls. E75 induced a higher Bcl-xL to Bad ratio than S5K. A7.3 and F8-1 variants induced even higher Bcl-xL to Bad ratios than E75, indicating that their effects were sequence-specific S5K was a slightly stronger up-regulator of Bcl-2 than E75. The inhibitory effects of E75 and S5K on Bad up-regulation were similar, although E75 was a slightly stronger inhibitor. **These results indicate that E75-mediated protection from CD95-mediated apoptosis of S5K-CTL correlated with down-regulation of proapoptotic family members.**

The increase in the level of expression of Bcl-2 was considered significant compared with the up-regulation of Bcl-2 induced by a mitogen (PHA) in the same cells for 96 h. This is evident when the Bcl-2 and Bcl-xL to actin ratios are compared at stimulation with S5K and PHA vs the Bcl-2 and Bcl-xL to actin ratios in unstimulated cells For S5K stimulation, the ratios are 1.72 (Bcl-2) and 1.32 (Bcl-xL), while for PHA stimulation the ratios are 1.55 (Bcl-2) and 4.37 (Bcl-xL). The increase in the levels of

Bcl-2 and Bcl-xL at stimulation with PHA is comparable with the increase reported in other studies in the presence of a mitogen, but in the absence of IL-2. Increase in the Bcl-2 levels is in general observed if mitogen-activated T cells are given high doses of IL-2. Thus, activation and expansion of tumor-reactive CTL by the variant S5K allowed better survival of these CTL in response to the wild-type tumor Ag.

Once S5K-CTL were established and were protected from apoptosis by restimulation with S5K, signals from the wild-type E75, or variants with Ala^{7} side chain extended with 3CH2 groups, or Phe^{8} with side chain shortened with 1CH2 group, induced even higher Bcl-xL:Bad ratios. In S5K-CTL, E75 also increased the levels of TCR expression and Bcl-2 expression more than S5K. Considering that S5K was recognized with lower affinity than E75 by E75-induced CTL, it is possible that S5K is a weak CTL activator similar to homeostatic inducers .A possible explanation for the low affinity of S5K-CTL for E75 is that the stimulus is not sufficiently strong to bring TCR together in the appropriate conformation for wild-type Ag recognition. This may have the advantage of extending the life of such CTL. Further studies with distinct agonists should address this question.

One important consideration now emerging from lymphocyte activation studies is that the CTL response to an Ag first expands and then contracts to bring down the number of activated effectors( ) Reduction in the number of activated CTL is initiated by Ag and manifests by induction of apoptosis at restimulation a phenomenon that is amplified by IL-2. The development of agonistic variants that more strongly activate antitumor effector CTL is a necessary requirement for immunotherapy

Fine-tuning of activation of tumor-reactive CTL by weak agonists, designed by molecular modeling, may circumvent cell death or tolerization induced by tumor Ag, and thus, may provide a novel approach to the rational design of human cancer vaccines.

3. N-terminally extended HER-2 peptides which activate memory hi-av E75-TCR ^{hi} CD8 $^{+}$ cells.

We quantified the cellular immune responses to HER-2 peptides **(Table 1)** of 14 breast cancer patients,(BCP) whose tumors were either $HER2^{hi}$ or $HER2^{lo}$. The frequency of IFN-γ responses of PBMC cultures to each of the peptides in this study demonstrated that responses to the *protected* LRMK-HER-2 (774-779) were significantly higher in magnitude compared to 4 other protected LRMKAva-HER-2 peptides or to *protected* peptide LRMKAva-HER-2(774-789).

Because protected HER-2 (776-789) and LRMK-HER-2 (776-789) were not available we could not conclude that *protected* Ii-HER-2(774-779) is a more efficient immunogen than these HER-2 peptides in similar form. The mixtures of *protected* LRMK-HER-2 (774-779) and LRMKAva-HER-2(776-779) with E75, respectively, expanded higher numbers of $E75\text{-}TCR^{hi\ +med}$ $Perforin^{+}$ $CD8^{+}$ cells than the other by LRMKAva-HER-2 peptides or E75 alone. In the absence of Ava –conjugated HER-2 peptides we cannot conclude that the immunogenicity of HER-2(774-779) is enhanced by exclusion of Ava and that the immunogenicity of HER-2(776-789) is enhanced by Ava.

We do not have yet an explanation for this finding. It is possible that the lifting of the fractured horizontal plane optimized the contact of the LRMK with TCR. Since the LRMKAva-V+F7 was inactive, and the LRMKAva-GV+F7 was also inactive, then the di-peptide GV, and the amino acid Ava had similar effects in enhancing the contact of LRMK with T cell receptor. The shift of the plane is more likely upwards because Ava is followed by the horizontal plane formed by the LRMK peptide bond. This conclusion is supported by our earlier results from the laboratory of Dr. Ioannides. Earlier studies found that PBMC from a healthy individual and lymph node lymphocytes from a BCP responded with more IFN-γ and less IL-4 respectively, to LRMK-HER-2 (777-790) (33). However, LRMK extended with other amino acids of the Ii molecule was more toxic than the Ii-tetrapeptide. Additional studies are needed to define LRMK-activity relationships and their significance.

These studies expanded our original findings on T cell activation by Fisk and Tuttle et al. (34,35). Fisk et al. (34) performed the first extensive study with seven distinct HER-2 peptides in 25 ovarian cancer patients with advanced disease. They identified F7 (776-789) and F13 as the dominant naturally immunogenic peptides from the HER-2 molecules which activate T cells. Tuttle *et al.* previously demonstrated that PBMC from 13 of 18 BCP without disease after surgery, proliferated *in vitro* in response to a series of synthetic HER-2 peptides; cells responded most frequently to peptides G89 and F7 (35). G89 was recognized by T cells from 10 patients with the MHC class II HLA-DRβ1*04 haplotype, and less frequently by patients without that haplotype. IFN-γ secretion from two long-term T-cell lines derived from both a healthy donor and a patient was significantly greater in response to G89 than to several other peptides. Responses occurred in cells from both $HER2^{hi}$ and $HER2^{hi}$ patients. In several HER2- patients we also observed IFNγ secretion in response *to protected hybrid Ii-HER-2* peptides and to unprotected G89 (HER-2,777-790).

MHC-II bound peptides contain a core of nine amino acids which is extended with N-, and C-, terminal flanking residues. A large number of peptides eluted and sequenced from MHC-II molecules contain flanking residues of variable length. Our analysis of the predicted binding affinities with the program TEPITOPE( ), identified the sequence **YVSRLLGICL** as the core peptide of the MHC-II binding peptides . This peptide has one log higher affinity of binding for DR8 than for other MHC-II molecules. Extention of HER-2 (774-789) with LRMK does not increase the binding affinity of the "hybrid" peptide for any MHC-II molecules. These results support the novel hypothesis that LRMK points upward and not towards MHC pockets.

What is the possible mechanism of action of these peptides ? .

Figure 1 shows that LRMK-GV+F7 and GV+F7 induced similar proliferation and survival of the entire lymphocyte population with F7 and G89 at 0.7 and 3.5 uM. At 7.0 uM of each peptide LRMK-GV+F7 and GV+F7 induced death of 40% of PBMC while F7 and G89 expanded these PBMC, by 30-40%. This finding indicate that N-terminally extended F7 had a higher functional avidity for TCRs than F7. The dramatic decrease in numbers at 7.0 uM LRMK-GV+F7 and GV+F7 indicate that cells died by over-stimulation. Furthermore the massive death indicate that LRMK-GV+F7 and GV+F7 engaged TCRs of many specificities. Similar responses are frequently observed in response to bacterial superantigens, endotoxins. Endotoxins rapidly activate cytokine production by T cells, which die shortly thereafter.

Our data show that in $HER2^{hi}$ $HLA\text{-}A2^{+}$ patients studied, LRMK-HER-2 (774-789) and LRMK-Ava-HER-2 enhanced the proliferation of E75-$TCR^{Hi\ and\ med}$ CD8 cells. The mechanisms differed and are not elucidated. (This was not part of the Grant, we extended the studies in collaboration with AE. We received some funding. We plan to apply for joint patents.

Figure 2 shows that LRMK-HER-2(774-789) at 0.7μ M slightly increased the number of E75-TCR^{Hi} cells by 1.40 fold but decreased the number E75-TCR^{Hi} by 4.2 fold at 3.5 μM. In contrast, LRMKAva-HER-2(776-789), increased the number of E75-TCR^{Hi} by 1.9 fold and decreased the number of E75-TCR^{Hi} by only 1.4 fold. LRMKAva-HER-2(776-789), increased the number of by 2.7 fold and decreased the number of by 1.3 fold at 0.7 μM and 3.5 μM respectively. Therefore the LRMK-Ava-HER-2 (776-789) at low 0.7 μM was the most effective "*helper* " of existent memory hi-av E75-TCR^{Hi} $CD8^{+}$ cells. (Figure not added because of issues of Intellectual Property)

When both E75 and "helper HER-2 peptides were used together we found that LRMK-HER-2(774-789)-provided help resulted in expansion of more E75-TCR^{Hi} $CD8^{+}$ cells at 0.7 μ M , while LRMK-Ava-HER-2 (776-789) provided more help at 3.5 μM. These results are important in light of dose-

escalation and repeated vaccination protocols used to expand hi-av cells. Increase in the amount of the helper peptide will have negative effects on patients.

Surprisingly, at 7 μM LRMK-GV+F7 and GV+F7 decreased the number of cells with T^{reg} phenotype more than the other HER-22 peptides. The hypothesis that elimination of T^{reg} expanded better $CD8^+$ cells it is not supported by our data. At concentrations between 7 and 20 μM LRMK-Her-2 (774-789) induced death of CD8+ cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. Novel immunogenic variants of tumor Ag (TA) can be designed and synthesized with the aid of modeling of 3-D structures of peptide MHC-I complexes.
2. The novel immunogenic variants are generated with emphasis on the side chains of the tumor Ag which point upwards, towards the T cell receptor. The main positions are $G^{372,}$ A^{375} and F^{376}
3. The strenght of stimulation by these novel antigenic variants can be finely tuned by decreasing the distance between the side chain and T cell receptor.
4. The strenght of stimulation follows a bell-shaped plot . Variants with stronger stimulatory function than optimal induce death.
5. Novel antigenic variants of weaker stimulatory function should be significant for survival of T cells activated by stronger agonists at priming.
6. Novel antigenic variants with extended the N-terminus are stronger stimulators of "help" for survival of existent hi-av CD8 + T cells. Others are strong activators of *"help"* for activation of naïve cells by the MHC-I peptide complexes. These effects are evident at low concentrations of "helper 'peptides.

7. The strong stimulatory potency may result in early death of responders. Although the complete mechanism has not been elucidated the response may resemble that of super-antigens.

8. The activating function for these variants depends on the absence and presence of the group G^{774} V^{775} or of the un-natural amino acid 5-amino butyric at the N-terminus of the helper HER-2 peptide.
9. Survival of variant-activated T cells depends on the higher ratios of BCL-2 and BCL-xL to Bax and Bad and not only on increase in BCL-2.

10. Activation of cell death pathways by variants is through TCR activation of Caspase-9 and not caspase-3.

10 The variant immunogens can be expressed in the mRNA of influenza virus proteins such as neuraminidase. (37,38)

11. The use of HAB, together with caspase-9 inhibitors and IL-2Rβ chain (CD122) blockers should be useful to expand TCR^{hi} cells in large numbers for adoptive biotherapies.

REPORTABLE OUTCOMES:

1. Novel immunogenic variants of tumor Ag (TA) can be designed with focus on the side chains of the HER-2 tumor Ag, E75 which point towards the T cell receptor. The main positions are G ^{372,} A^{375} and F ^{376}
2. The strenght of stimulation by these novel antigenic variants can be finely tuned by decreasing the distance between the side chain and T cell receptor.
3. Novel antigenic variants with the N-terminus extended help expansion of hi-av CD8+ cells . Although the complete mechanism has not been elucidated the response may resemble that of super-antigens.
4. The use of HAB, together with caspase-9 inhibitors and IL-2Rβ chain (CD122) blockers should be useful to expand TCR^{hi} cells in large numbers for adoptive biotherapies.
5. The variant immunogens can be expressed in the mRNA of influenza virus proteins such as neuraminidase. (37,38)

DO NOT DISCLOSE THIS YET.

1. Vectors based on fusion proteins made by tumor antigens and small proteins proteins from small viruses cold be the next generation of vaccines.
2. Disabled small RNA viruses such as influenza expressing novel immunogenic variants can be the next generation of vaccines. However these studies require quarantine before any consideration.
3. Natural small RNAs from influenza virus or designed microRNA together with novel immunogenic variants can be enginered in novel Immuno-stimulatory multi-molecular complexes which can vaccinate patients without concerns of viral infection.

CONCLUSIONS

These results suggest that changes in ligand affinity for TCR by substitutions with natural amino acids will be never able to modulate the weak forces executing differential control of effector gene expression in TCR^{hi} cells. Differences of only **two** methylene groups (e.g., Gly vs γ-aminobutyric acid) are absent in natural amino acids; also, differences of **one** CH_2 group in linear chains of three to four CH_2 groups are not present in natural amino acids. The side chains of Val and Leu/Ile differ in one amino acid length and are branched. Differences in the van der Waals forces from one CH_2 group in Ser and Thr should be masked by the 10-fold stronger forces from -OH groups.

Synthetic amino acids are not genetically encoded; although some are generated in humans by enzymatic reactions during metabolism (e.g. γ-aminobutyric acid, a neuro-transmitter). They are not known to be incorporated into proteins. Expression of synthetic amino acids in proteins suggests that posttranslational modifications, such as methylation/ demethylation of a CTL epitope, may be taking place if the corresponding bacterial enzymes are present (36). The presence of norvaline and norleucine in bacterial proteins, in recombinant proteins, and in antibiotics, raises the possibility that TCR^{hi} cells specific for self-Ag are periodically activated and inactivated after interactions with bacterial or fungal pathogens.

In summary, our results provide a novel basis for possible control of proliferation and terminal differentiation of human anti-tumor CTLs that recognize self-Ag. The sensitivity to small changes in atomic force demonstrated by different responses to a 1- to 2-CH_2 difference in the Ag will be useful in the induction of anti-tumor responses.

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Abstracts

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17.Chang DZ, Tsuda N, Abbruzesse, J and Ioannides, CG. Annual Meeting of the Society of Biological Therapy.Alexandria, VA, November,2005.

2006, is not complete and complete information is not available yet.

Novel Patents and and Licence applications.

1.Novel melanoma peptide vaccines derived from the high molecular weight proteo-glycan antigen. **Ioannides, C.G.,** Kawano, K, Ferrone S., Murray, J.L. *Invention Disclosure Report, IDR: 242-02 submitted February 2004.Provisional patent submitted November 2004 by Roswellpark Memorial Institute. Buffallo, New York.*

2.Induction of tumor immunity by controlled modulation of amino acid side chain length using methl/methylene (CH3/CH2) groups. Campbell, M.A., O'Brian, C.A., Carter, D., **Ioannides, C.G.** *Invention Disclosure Report, submitted March 2001. Provisional patent submitted March 2002.Complete patent application Submitted 2003.(part of the consortium agreement with DOD for APT) .*

***3.** Invention Disclosure Report. Anti-tumor synthetic designer RNAs. Ioannides CG, Tsuda, N, Kawano, K and Chang , DZ, March 2006. Provisional Patent Submisison pending.*

4. Invention Disclosure Report. Immunity by HER-2 peptides extended with Ii-tetrapeptide-LRMK.

Impact of patent and license applications

1. Succesful work on this grant application resulted in two novel patent applications. One is in collaboration with COL. DR.George Peoples, WRAMC. (george.peoples@na.amedd.army.mil). We formed a new Biotechnology company for development of cancr vaccines. 25% of this new company is owned jointly by MDACC and Department of Defense, as a consequece of the collaboration on this grant**.** Therefore we not only spent money but found ways to bring money back to the DOD.

2. There are intelectual properties issues with Generex, because of an ongoing collaboration with its subsidiary Antigen Express. Based on our finding in previous DOD grants, that HER-2 peptide F7 acivates T cells with Dr.Bryan Fisk, (bryan.fisk@na.amedd.army.mil), Antigen Express designed extended F7 peptides. We tested these peptides with our methodology, of our design, and identiffed the candidate active helper peptides. One is active at low concentration during priming activation of E75-TCR $^{+}$ cells. The other is more active on memory E75-TCR $^{+}$ cells when they are not stimulated by Antigen/Vaccine. Our oppinion is that the Invention belongs jointly to all of us and it is not exclusive property of Generex Corporation . We hope that ongoing negociations will result in funding and royalties for DOD.

Funding received based on work suported by this award.

1.*Licensing Agreement: MDAnderson Cancer Center-Henry Jackson Foundation Partners with Advanced Peptide Therapeutics, Scottsdale, AZ*. Co-Inventor, Laboratory Director and Pro-bono Project Manager on behalf of all co-inventors. Up-Front Payments $ payable in 5 years, Licensing fee (Earnest Money), $. paid on 10/20/2006. Additional payments are a minimum of $. Project manager from OTC-MDACC: Dr.Philip Thomson, 713-745-9624) should be contacted for verification of details.

2.Aventis-Pasteur Corporation: Characterization of the TRPS-1 gene in breast cancer: Role in tumorigenesis and potential as a breast cancer vaccine target. Jan 1, 2006 – Dec 31, 2008. Direct costs: $, Total costs: $. Dr. Laszlo Radvanyi *Principal Investigator,* Constantin G.Ioannides, PhD, *Co-Investigator* and *Principal Investigator Aim-4*. Total direct funding for Aim-4, $. (Payments pending)

3.SPORE in Pancreatic Cancer: Micro-RNA-targeting of GLI-1 for inhibition of pancreatic tumor growth. *Principal Investigator* (Year 1):10/2005-9/2006. Direct paid $.This program will expand and the payments will continue.

4.Collaborative Research Agreement with Antigen-Express Corporation. 9/1/03-Present. Dr. James L. Murray, *Principal Investigator*, Dr.Constantin G. Ioannides, *Co-Principal Investigator*. Induction of immunity to breast cancer by hydrophobically appended variants of the HER-2 CTL epitopes in synergy with Ii-key hybrids of the HER-2 helper epitope.Total: $, Total direct funding for Dr. Ioannides laboratory: $.

7.Bristol Myers Corporation: Hydrophobically Appended Antigens in Breast Cancer. Through Dr. Moshe Talpaz. 6/2002 - 9/2003.Total direct direct funding for Dr. Ioannides' Laboratory. $.

8. SBIR, Antigen Express Corporation: Her-2/neu T helper memory vaccine for breast cancer. Robert E.Humpreys, MD. PhD, *Principal Investigator* 2002 – 2005. Constantin G. Ioannides *Collaborator* .The SBIR provided direct funding through a Collaborative Research Agreement).

8. Melanoma SPORE. P50 CA093459-01A1. 2004-2008. (Elizabeth A. Grimm, Principal Investigator) Constantin G. Ioannides, *Co-Investigato r* (5% effort funded) in Project 3: Class II Melanoma Vaccine.

(Jeffrey E. Lee. *Principal Investigator,* Constantin G. Ioannides, *Co-Investigator* (5% effort funded) National Institutes of Health. 2003-2007. Total: $11,000,000.

9.Research Grant: (*Principal Investigator*) Induction of tumor immunity using novel recombinant temperature-sensitive influenza viruses as vectors. Keck Foundation. 11/01/02-Present. External Peer-Reviewed. Total direct $50,000.

Grant applications unfunded and pending are not included.

Personnel supported by this study:

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1. Constantin G.Ioannides, PhD, Professor
2. Clayton Efferson, Msc, Senior Research Assistant
3. Kouichiro Kawano, MD. PhD Post-Doctoral Fellow,
4. Naotake Tsuda, MD, PhD Post-dctoral Fellow
5. Shankhar Sellapan, BSC Graduate Student. Graduate 2005
6. John D.Davis, Bsc Graduate Student , Graduated 2005.
7. Yufeng Li, Bsc, Msc, ----------Graduate Student, Started Fall 2005.

Employement and carrer development of the personnel who worked on this Grant.

1. Clayton Efferson,PhD --→ Research Scientist ,Merck, Co, Boston, MA
2. Shankahar Sellapan PhD→ Research Scientist, San Antonio, Tx,
3. John D.Davis, PhD -→ Research Scientist Houston,

4. Kouichiro Kawano, MD, PhD→ Awarded tenure-track, Department of Gynecology, University of Kurume , Japan.

5. Zavid Z.Chang, MD, PhD, -→Awarded Tenure Track, Department of GI-Medical Oncology, MDAnderson Cancer Center

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2 PhD degree. Drs. Sellapan and Davis.

CONCLUSIONS

Tumor antigens are weak immunogens compared with viral and bacterial antigens. They are in fact self-antigens. Enhancement of tumor antigens immunogenicity has not been yet accomplished with conventional approaches. In fact, conventional approaches such as enhanced co-stimulation or inhibition of [inhibition of co-stimulation] or escalating doses of IL-12 resulted in life-treatening effects for volunteers and patients. The negative effects are due to paroxistical amplification of auto-immunity against healthy tissues.

These results suggest that changes in ligand affinity for TCR by substitutions with natural amino acids will be never able to modulate the weak forces executing differential control of effector gene

expression in TCR^{hi} cells. Differences of only two methylene groups (e.g., Gly vs γ-aminobutyric acid) are absent in natural amino acids; also, differences of one CH_2 group in linear chains of three to four CH_2 groups are not present in natural amino acids. The side chains of Val and Leu/Ile differ in one amino acid length and are branched. Differences in van der Waals forces from one CH_2 group in Ser and in Thr should be masked by the 10-fold stronger forces from -OH groups.

Synthetic amino acids are not genetically encoded; although some are generated in humans by enzymatic reactions during metabolism (e.g. γ-aminobutyric acid, a neuro-transmitter). They are not known to be incorporated into proteins. Expression of synthetic amino acids in proteins suggests that posttranslational modifications, such as methylation/ demethylation of a CTL epitope, may be taking place if the corresponding bacterial enzymes are present (36). The presence of norvaline and norleucine in bacterial proteins, in recombinant proteins, and in antibiotics, raises the possibility that TCR^{hi} cells specific for self-Ag are periodically activated and inactivated after interactions with bacterial or fungal pathogens.

In summary, our results provide a novel basis for possible control of proliferation and terminal differentiation of human anti-tumor CTLs that recognize self-Ag. The sensitivity to small changes in atomic force demonstrated by different responses to a 1- to 2-CH_2 difference in the Ag will be useful in the induction of anti-tumor responses.

This study successfully demonstrate that if the amplification is focused on cells with a defined specificity, which function as effectors of tumor death, a enhancement of T cell activation can be achieved. The hydrophobically appended antigens can enhance or attenuate activation of effectors.

Because they are modified only in the chains which point towards the T cell receptor, the expansion of cross-reactive T cells is limited. Furthermore, because they are based on side chain modifications of un-natural amino acids, they advance an universal approach applicable to all tumor antigens in all cancers. Last, this approach appears to be useful in control of proliferation and differentiation of regulatory T cells.

The knowledge will result in a medical product. The product vaccine will be important for personalized cancer therapies. We will test what the immune responses of the cancer patient *in vitro* before vaccination . We will identify most efficient activators over time. We will be able to enhance or to attenuate responses of the patient vaccine. Currently we can increase the vaccine dose and hope that one cancer vaccine suits all. The speed with which our results were brought for commercialization demonstrated their potential significance for cancer treatment.

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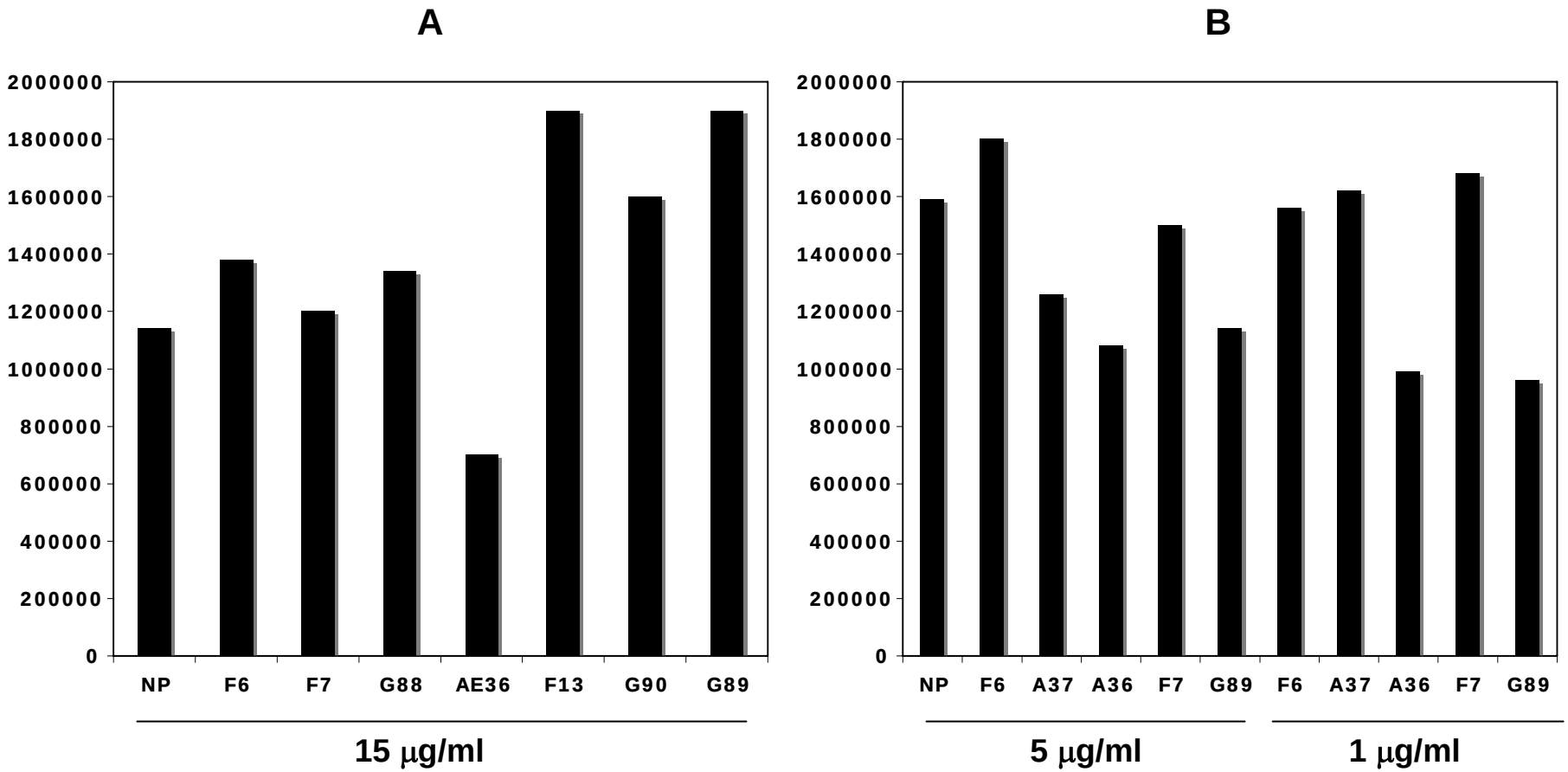
Figure 1


A
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F6
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G88
AE36
F13
G90
G89
15 μg/ml
B
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1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
NP
F6
A37
A36
F7
G89
F6
A37
A36
F7
G89
5 μg/ml
1 μg/ml

Table I. HER2 peptides extended at N-terminus with the tetrapeptide LRMK from the Ii - invariant chain with the intercalated the un-natural amino-acid Ava.

Number	N-term	Sequence	N-ext.	Immune Responses	
				Ex Vivo[a]	After Immunization[b]
GV+F7	774	G**V**GSP**YV**SRLGICL	0	No	Yes
G89 (F7-2)	777	SP**YV**SRLGICLT	0	Yes	ND
GV+F7	776	G**V**GSP**YV**SRLGICL	LRMK (#)	This study	(31, 35)
GV+F7	776	G**V**GSP**YV**SRLGICL	LRMK- Ava (**)	This study	ND
V+F7	775	**V**GSP**YV**SRLGICL	LRMK-Ava	This study	ND
F7	776	GSP**YV**SRLGICL	LRMK-Ava	This study	ND
F7-1	777	SP**YV**SRLGICL	LRMK-Ava	This study	ND

(*)All peptides containing LRMK were designed by Dr.Robert E.Humphreys, AE(now Generex). as "protected' .HER2 Peptides marked with asterisk were : N-terminally acetylated and C-terminally amidified. (**) indicate 5-NH2-butyric acid (*ava = a valeric acid)*. Peptide G89/F7-2 (777-790) was synthesized by MDAnderson Cancer Center Core laboratory as free peptide.

All other peptides were synthesized by Antigen Express. Candidate position 1 anchors (P1 anchors) are bolded.

(#) LRMK are the amino acids 79-82 of the Ii protein from the human and murine Ia chain precursor.